Adrenomedullin and Nitric Oxide Inhibit Human Endothelial Cell Apoptosis via a Cyclic GMP-Independent Mechanism

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Abstract—Adrenomedullin, which was discovered as a vasodilating peptide, has been reported to be produced in various organs, in which adrenomedullin regulates not only vascular tone but also cell proliferation and differentiation in an autocrine/paracrine manner. We evaluated the effect of adrenomedullin on endothelial cell apoptosis. Human umbilical vein endothelial cells underwent apoptosis when cultured in serum-free medium. Treatment with adrenomedullin reduced the number of cells with pyknotic nuclei (Hoechst 33258 staining) and inhibited cell death (dimethylthiazol-diphenyltetrazolium bromide assay) in a dose-dependent manner. The administration of adrenomedullin did not alter the expression levels of Bcl-2 family proteins. Experiments with analogs of cAMP or a cAMP-elevating agonist demonstrated that elevation of the intracellular cAMP concentration does not mediate the antiapoptotic effect of adrenomedullin. The coadministration of N-nitro-L-arginine methyl ester (2 mmol/L), an inhibitor of nitric oxide synthase, abrogated the effect of adrenomedullin. Lower doses of sodium nitroprusside (1 to 10 μmol/L), a nitric oxide donor, mimicked the antiapoptotic effect of adrenomedullin. The antiapoptotic effect of sodium nitroprusside was not attenuated by the inhibition of soluble guanylyl cyclase with 1 μmol/L oxadiazolo-quinoxalin-1-one nor could apoptosis be inhibited by the incubation of human umbilical vein endothelial cells with 1 mmol/L 8-bromo-cGMP, a cell-permeant cGMP analog. These results indicate that adrenomedullin and nitric oxide inhibit endothelial cell apoptosis via a cGMP-independent mechanism. (Hypertension. 2000;36:83-88.)

Key Words: endothelium ■ adrenomedullin ■ apoptosis ■ nitric oxide ■ cyclic AMP

Adrenomedullin is a hypotensive peptide that was identified recently.1 Accumulating evidence suggests that adrenomedullin has more biological effects than initially expected as a vasodilating reagent.2 Adrenomedullin production has been detected in various organs,3,4 where it regulates not only vascular tone but also proliferation, differentiation, and migration of various cell types, including smooth muscle cells5,6 and mesangial cells.7 These findings suggest that adrenomedullin contributes to physiological cell turnover and tissue remodeling in an autocrine/paracrine manner.

On the other hand, the plasma concentration of adrenomedullin has been reported to be elevated in pathological conditions such as hypertension,8 heart failure,9,10 and renal failure.11 A marked increase in plasma adrenomedullin concentration was observed in patients with septic shock.12 These clinical observations have led to 2 opposite hypotheses regarding the roles of adrenomedullin under pathological conditions.

First, adrenomedullin may contribute to the pathogenesis of the diseases. Adrenomedullin production by vascular cells is markedly upregulated by tumor necrosis factor-α, interleukin-1, or lipopolysaccharide (LPS) in vitro.13 Adrenomedullin also has a synergistic effect on cytokine production by monocytes/macrophages and fibroblasts induced by tumor necrosis factor-α, interleukin-1 or, LPS.14 Thus, adrenomedullin upregulation may play a role in the exacerbation of inflammatory response. Furthermore, elevated levels of adrenomedullin may lead to severe hypotension during endotoxin shock.

Second, adrenomedullin induction could be a self-defense response against pathological stimuli. Systemic vasodilatation induced by adrenomedullin would ameliorate the clinical course of hypertension and heart failure. Adrenomedullin could also increase the blood supply to injured tissues. Recently, it was reported that transgenic mice that overexpress adrenomedullin were resistant to LPS-induced shock.
and tissue injury,\textsuperscript{15} supporting the idea of a protective role for adrenomedullin under pathological conditions. However, the precise mechanism remains to be elucidated.

Here, we show that adrenomedullin protects human endothelial cells from apoptosis induced by serum starvation. Results indicate that the antiapoptotic effect of adrenomedullin is mediated by the stimulation of NO production by endothelial cells. Our findings propose a novel biological action of adrenomedullin and may explain the functional significance of adrenomedullin upregulation under pathological conditions.

**Methods**

**Cells and Reagents** Human umbilical vein endothelial cells (HUVECs) were isolated as previously described\textsuperscript{16} and cultured in EGM-2 (Clonetics), containing 2% FBS, 10 ng/mL human epidermal growth factor, 1.0 μg/mL hydrocortisone, and 12 μg/mL bovine brain extract. Cells from up to the eighth passage were used for this study. Human adrenomedullin was purchased from Peptide Institute Inc. Human recombinant vascular endothelial cell growth factor (VEGF\textsubscript{165}) was purchased from R&D Systems. N\textsuperscript{\textsubscript{-}}Nitro-l-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), forskolin, 8-bromo-cAMP (8-Br-cAMP), dibutyryl-cAMP, 1H-1,2,4-oxadiazolo(4,3-a)quinazolin-1-one, 8-bromo-cGMP (8-Br-cGMP), and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma Chemical Co.

**Serum Depletion and Morphological Examination** Human umbilical vein endothelial cells (HUVECs) cultured on a 24-well plate were washed twice with PBS and then cultured in serum-free EBM (Clonetics) for 24 or 36 hours in the presence or absence of adrenomedullin. Cells were fixed in 4% formaldehyde, stained with Hoechst 33258, and observed under a microscope equipped with phase-contrast and epifluorescence optics (Olympus).

**Cell Viability Assay** HUVECs were subcultured on a 96-well plate and incubated in serum-free EBM for 48 hours in the presence or absence of adrenomedullin. Cell viability was measured by the MTS (dimethylthiazol-diphenyltetrazolium bromide) assay,\textsuperscript{17} and percent cell death was calculated as \(100\times\frac{1}{1+\text{viability of treated endothelial cells/viability of untreated endothelial cells}}\).

**Immunoblotting** HUVECs were cultured in serum-free EBM in the absence or presence of adrenomedullin for 24 hours and lysed with a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 mmol/L PMSF in PBS. The protein content was measured with BCA protein assay reagent (Pierce Chemical Co). The cell lysates (15 μg/lane) were analyzed by SDS-PAGE with a 10% polyacrylamide gel and were transferred to a polyvinylidene fluoride membrane (Millipore). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS), the membrane was incubated with anti-Bcl2 (Transduction Laboratory), anti-Bcl-x (Transduction Laboratory), and anti-Bax (Santa Cruz Biotechnology) antibodies. Membranes were washed in TBS supplemented with 0.1% Tween 20 (T-TBS) and incubated with horseradish peroxidase–conjugated sheep antibody to mouse Ig or rabbit Ig (1:3000; Amersham). After washing in T-TBS, antibody binding was detected with an enhanced chemiluminescence system (Amersham).

**NO Measurement** NO production from endothelial cells was measured with the use of diaminofluoresceins as reported elsewhere.\textsuperscript{18} HUVECs were cultured in EBM containing 7 μmol/L diaminofluoresceins in the presence or absence of adrenomedullin (10\textsuperscript{\textsuperscript{\textsuperscript{-7}}} mol/L) or SNP (10 μmol/L) for 6 hours. Medium was collected and fluorescence intensity was measured at 375 nm excitation and 425 nm emission.

**Radioimmunoassay for Adrenomedullin** Adrenomedullin concentration in the culture medium was measured by means of radioimmunoassay as previously described.\textsuperscript{19} HUVECs were cultured on a 24-well plate in EGM-2. The medium was replaced with serum-free medium and incubated at 37°C in 5% CO\textsubscript{2} in the presence or absence of interleukin-1β for 3 hours, after which the medium was collected and kept in –80°C. Culture medium was concentrated with the use of Sep-Pak C18 cartridges and then dissolved in 100 μL radioimmunoassay buffer consisting of 0.05 mol/L sodium phosphate (pH 7.4), 1% BSA, 0.5% Triton X-100, 0.08 mol/L NaCl, 0.025 mol/L EDTA-2Na, and 0.05% sodium azide. After the medium was mixed with 50 μL 12\textsuperscript{i}I-labeled ligand and 50 μL antiserum at dilutions of 1:182 and 1:250, the mixture was incubated at 4°C for 24 hours. The incubation was stopped by the addition of 50 μL of 1% bovine γ-globulin and 500 μL of 24% polyethylene glycol. After vigorous shaking, the mixture was incubated at 4°C for 20 minutes and centrifuged at 2000g at 4°C for 30 minutes. The supernatant was aspirated, and radioactivity in the pellets was counted in a γ-counter.

**Statistical Analysis** Data are expressed as mean±SEM. Statistical comparisons were made with ANOVA followed by the Student-Newman-Keuls test. Differences with a \(P\) value of <0.05 were considered statistically significant.

**Results**

**Adrenomedullin Inhibits Endothelial Cell Apoptosis Induced by Serum Starvation** HUVECs cultured in serum-free medium displayed an apoptotic morphology characterized by cell shrinkage (Figure 1A) and nuclear condensation (Figure 1B). When cultured in the presence of 10\textsuperscript{\textsuperscript{-6}} mol/L adrenomedullin, the number of cells that show the apoptotic phenotype significantly decreased. HUVEC apoptosis was also confirmed by a decrease in cell viability, another indicator of apoptotic cell death, which was assessed with the MTS assay.\textsuperscript{20} Adrenomedullin inhibited endothelial cell death in a dose-dependent manner (Figure 1C).

The production rates of endogenous adrenomedullin in the culture medium of untreated HUVECs was 2.9±0.3 fmol \cdot mL\textsuperscript{\textsuperscript{-1}} \cdot 24 h\textsuperscript{-1}. Treatment with interleukin-1β (5 to 20 ng/mL) upregulated the adrenomedullin production by 12- to 113-fold.

**Adrenomedullin Does Not Change Levels of Bcl-2 Family Proteins** Next, we studied the mechanism by which adrenomedullin inhibits endothelial cell apoptosis. The Bcl-2 family of proteins functions as positive and negative regulators of apoptosis.\textsuperscript{21} VEGF and basic fibroblast growth factor are known to inhibit endothelial apoptosis by changing the levels of the Bcl-2 family proteins.\textsuperscript{22,23} In the present study, VEGF (50 ng/mL) markedly upregulated Bcl-2 expression in serum-starved HUVECs (Figure 2). However, treatment with adrenomedullin had no effect on the expression of the proapoptotic protein Bax or the antiapoptotic proteins Bcl-x and Bcl-2.

VEGF is also known to promote endothelial cell survival by activating the phosphatidylinositol kinase/Akt signal...
transduction pathway.\textsuperscript{24,25} Immunoblotting with antiphosphorylated Akt antibody revealed that the treatment of HUVECs with adrenomedullin for 12 hours had no effect on the phosphorylation of Akt, whereas VEGF (50 ng/mL) significantly promoted the phosphorylation of Akt (data not shown).

Effect of Adrenomedullin Is Not Mediated by cAMP
The vasodilating effect of adrenomedullin is known to be mediated by an increase in intracellular cAMP.\textsuperscript{1} cAMP has been shown to block serum deprivation–induced apoptosis in some cell types.\textsuperscript{26} Therefore, we hypothesized that cAMP may mediate the antiapoptotic effect of adrenomedullin. Treatment with analogs of cAMP (0.5 mmol/L dibutyryl-cAMP or 0.5 mmol/L 8-Br-cAMP) or a cAMP-elevating agonist (10^{-6} mol/L forskolin) failed to prevent endothelial cell death induced by serum deprivation (Figure 3), indicating that cAMP is not a major mediator of the antiapoptotic effect of adrenomedullin on endothelial cells.

Antiapoptotic Effect of Adrenomedullin Is Mediated by NO
Recent reports have indicated that the vasodilating effect of adrenomedullin is also mediated by NO released from endothelial cells, which suggests that NO may transmit the biological effects of adrenomedullin.\textsuperscript{27} When adrenomedullin was added to the cultures together with 2 mmol/L L-NAME, an inhibitor of NO synthase, the antiapoptotic effect was completely abrogated (Figures 4A and 4B). Furthermore, SNP (10 μmol/L), a NO donor, mimicked the antiapoptotic effect of adrenomedullin.

We directly measured NO produced by endothelial cells by adding a fluorescent indicator to the culture medium that contained effective doses of adrenomedullin or SNP. The fluorescent intensity in the medium containing 10^{-7} mol/L SNP (Figure 5) was significantly higher than that with adrenomedullin or 8-Br-cAMP (Figure 6).

**Figure 1.** Adrenomedullin inhibits endothelial cell apoptosis. A, The apoptotic phenotype was reversed by adrenomedullin. HUVECs were cultured on a 24-well plate, incubated in serum-free medium in the absence or presence of adrenomedullin (10^{-6} mol/L) for 36 hours, and observed under a microscope equipped with phase-contrast optics. B, Nuclear pyknosis was reversed by adrenomedullin. HUVECs were cultured in serum-free medium in the absence or presence of adrenomedullin for 24 hours. Cells were fixed in 4% formaldehyde, stained with Hoechst 33258, and observed under a microscope equipped with epifluorescence optics. C, Dose-dependent inhibition of endothelial cell apoptosis by adrenomedullin. HUVECs were cultured on a 96-well plate and incubated in serum-free medium for 48 hours in the absence or presence of adrenomedullin as indicated. Cell viability was determined with the MTS assay, and percent cell death was calculated as described in Methods. *P<0.05 vs control.

**Figure 2.** Adrenomedullin does not alter the levels of Bcl-2 family proteins. HUVECs were cultured in serum-free medium in the presence or absence of adrenomedullin (AM 10^{-8}, 10^{-7}, or 10^{-6} mol/L) or VEGF (50 ng/mL) for 24 hours. Cell lysates (15 μg) were loaded onto an SDS–10% polyacrylamide gel and analyzed by Western blotting with the indicated antibodies.

**Figure 3.** cAMP is not a major mediator of the antiapoptotic effect of adrenomedullin. HUVECs were cultured on a 96-well plate and incubated in serum-free medium for 48 hours in the absence or presence of adrenomedullin (10^{-6} mol/L), dibutyryl-cAMP (0.5 mmol/L), 8-Br-cAMP (0.5 mmol/L), or forskolin (10^{-6} mol/L). Cell viability was determined with the MTS assay, and percent cell death was calculated as described in Methods. *P<0.05 vs control.
adrenomedullin was significantly higher than that in the control medium (33.1 ± 0.7 versus 28.8 ± 1.4, P < 0.05). SNP at 10^−6 mol/L also increased the intensity to a level as high as 40.5 ± 1.0 (P < 0.01 versus control). These results indicate that NO mediates the antiapoptotic effect of adrenomedullin.

**Biphasic Regulation of Endothelial Cell Viability by NO**

Because it was reported that a biological effect of NO on myocardial contraction was concentration-dependently biphasic, we examined the effect of NO on endothelial cell viability in a wide range of concentrations of SNP (Figure 5A). At low concentrations (1 to 10 μmol/L), SNP inhibited the apoptosis in a dose-dependent manner. In contrast, at higher concentrations, SNP had a dose-dependent cytotoxic effect on endothelial cells. These results indicate that NO has a concentration-dependent biphasic effect on endothelial cell viability.

Next, we studied the mechanism of the antiapoptotic effect of NO. It is well known that cGMP and peroxynitrite mediate some of the biological functions of NO. However, treatment with 8-Br-cGMP, a cell-permeable cGMP analog, failed to mimic the antiapoptotic effect of NO (Figure 5B). Moreover, the coadministration of 1H-1,2,4-oxadiazolo(4,3-a)quinazolin-1-one, an inhibitor of soluble guanylyl cyclase, or SOD, a peroxynitrite scavenger, did not abrogate the antiapoptotic effect of NO, indicating that neither cGMP nor peroxynitrite mediates the antiapoptotic effect of NO.
thelial cells was shown to contribute to the vasodilating effect of adrenomedullin in vivo.27 Our results demonstrate that NO also mediates the antiapoptotic effect of adrenomedullin. We demonstrated that cGMP is not involved in the antiapoptotic function of NO, whereas the cGMP/cGMP-dependent kinase pathway mediates numerous biological functions of NO, including vasodilation and inhibition of vascular smooth muscle cell proliferation.32,33 Accumulating evidence postulates that NO prevents apoptosis induced with various stimuli by S-nitrosylating caspasases.34–38 Caspasases are intracellular cysteine proteases whose activation is required for the execution of apoptosis.39 NO production from endothelial cells and the consequent nitrosylation of caspasases can mediate the antiapoptotic effect of adrenomedullin.

Contrary to our observations and those of others,34–38 NO was demonstrated to promote apoptosis of various cell types, including glial cells,40 cardiac myocytes,41 vascular smooth muscle cells,42 and endothelial cells.43 In the present study, we found that NO acts as a biphasic regulator of apoptosis (ie, at low doses, NO inhibits apoptosis induced by serum deprivation, whereas at higher doses, it reduces the viability of cells). At low levels, NO seems to suppress the apoptotic pathway at multiple levels and via several pathways44,45 (ie, by inhibiting caspase activity through S-nitrosylation). However, at higher levels, NO may overwhelm cellular protective mechanisms and exert proapoptotic and cytotoxic effects. At high doses, NO and superoxide may also lead to the formation of peroxynitrite. Thus, we should be aware of the biphasic nature of the biological effects exerted by NO when we discuss the roles of NO under physiological and pathological conditions.

In the present study, the antiapoptotic effect of human adrenomedullin on HUVECs was not as potent as that of rat adrenomedullin on rat aortic endothelial cells.30 At a concentration as low as 10^{-10} mol/L, adrenomedullin significantly inhibited rat endothelial cell death induced by serum starvation. This difference appears to be due to a species-specific difference or a difference in the type of endothelial cells. In fact, there is much difference among species in the vasodilating effect, in cAMP production, and in the affinity of adrenomedullin.46

A significant antiapoptotic effect of adrenomedullin was observed at 10^{-7} mol/L. Baseline adrenomedullin levels in the culture medium of HUVECs were substantially low. However, interleukin-1β markedly increased adrenomedullin levels. These findings are consistent with previous reports.13,46 The adrenomedullin concentration at which we detected the significant antiapoptotic effect (10^{-7} mol/L) was much higher than that in culture medium or in human plasma. However, the local concentration of endogenous adrenomedullin that serves as an autocrine/paracrine might be much higher than that of secreted adrenomedullin.

In conclusion, we proved that adrenomedullin also works as a survival factor. The present results suggest that the marked upregulation of adrenomedullin under pathological conditions may function to defend tissues from various injurious agents in an autocrine/paracrine manner. Future studies with adrenomedullin-transgenic and/or knockout mice will further elucidate the roles of adrenomedullin in vivo.

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References


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